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Adverse effects of in vitro GenX exposure on rat thyroid cell viability, DNA integrity and thyroid-related genes expression☆,☆☆

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ABSTRACT

The hexafluoropropylene-oxide-dimer-acid (GenX) is a short-chain perfluoroalkyl substance that was recently introduced following the phase out of PFOA, as an alternative for the process of polymerization. GenX was detected at high concentrations in rivers, drinking water and in sera of exposed workers and recent findings suggested its potential dangerousness for human health.

Aim of the study was to assess the consequences of GenX exposure on in vitro thyroid cells with particular attention to the effects on cell-viability, proliferation, DNA-damage and in the thyroid-related genes expression.

FRTL-5 rat-thyroid cell line were incubated with increasing concentrations of GenX for 24 h, 48 h and 72 h to assess cell viability by WST-1. DNA-damage was assessed by comet assay and further confirmed by micronucleus assay. The proliferation of survived cells was measured by staining with crystal violet and evaluation of its optical density after incubation with SDS. Changes in TTF-1, Pax8, Tg, TSH-R, NIS and TPO genes expression were evaluated by RT-PCR.

GenX exposure reduced FRTL-5 viability in a time and dose-dependent manner (24 h: ANOVA $F = 22.286$; $p < 0.001$; 48 h: $F = 43.253$, $p < 0.001$; 72 h: $F = 49.708$, $p < 0.001$). Moreover, GenX exerted a genotoxic effect, as assessed by comet assay (significant increase in tail-length, olive-tail-moment and percentage of tail-DNA) and micronucleus assay, both at cytotoxic and non-cytotoxic concentrations. Exposure to GenX at concentrations non-cytotoxic exerted a significant lowering of the expression of the regulatory gene TTF-1 ($p < 0.05$ *versus* untreated) and higher expression of Pax-8 ($p < 0.05$ *versus* untreated) and a down-regulation of NIS ($p < 0.05$ *versus* untreated). In addition, cells survived to GenX exposure showed a reduced re-proliferation ability (24 h: ANOVA $F = 11{,}941$; $p < 0{,}001$; 48 h: $F = 93.11$; $p < 0.001$; 72 h $F = 21.65$; $p < 0.001$).

The exposure to GenX produces several toxic effects on thyroid cells in vitro. GenX is able to promote DNA-damage and to affect the expression of thyroid transcription-factor genes.

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1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are industrial chemicals developed both for manufacturing and customers products. Due to their chemical characteristics, PFASs earlier produced, such as perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) (and their precursors), are classified as persistent, bio-accumulative and/or toxic chemicals, and identified as possibly carcinogenic to humans (Austin et al., 2003; Coperchini et al., 2017; Domingo, 2012; Negri et al., 2017). Thus, The International Agency for Research on Cancer (IARC, 2017) and the US Environmental Protection Agency (EPA) released severe restrictions for their industrial use. For this reasons, different types of fluorinated-chemicals and new synthesis shorter chain compounds were developed as a progressively growing alternative ((IARC), 2017; Gordon, 2011; Heydebreck et al., 2015a, b; Wang et al., 2015). The PFAS compound hexafluoropropylene oxide dimer acid [(HFPO-DA), GenX] is used in the construction of high-performance fluoropolymers as a polymerization aid following the gradual elimination of PFOA(Beekman, 2016). GenX has been in use since nearly ten years (DuPont, 2017; Heydebreck et al., 2015a, b; Sun et al., 2019), with an annual production volume of 10–100 tons in Europe (DuPont, 2017; Pan et al., 2017). GenX was detected at high concentrations in river sampling sites both nearby and faraway from fluoro-chemichal plants, in drinking water and raw water of plants of drinking water treatment (Gebbink et al., 2017) (Brandsma et al., 2019; Hopkins et al., 2018), PFOA is still the PFAS most abundantly detected in the environment, both due to industrial emissions in the past and to the ongoing discharge and degradation of PFOA containing products. The global cumulative emission of PFOA in the environment was estimated to range from 2078 to 18,366 tons (Wang et al., 2014). Nevertheless, a recent study involving four European countries (United Kingdom, Neatherlands, Germany and Sweden) and two Asian countries (China and Korea) showed that GenX is frequently detected globally in surface waters, indicating ubiquitous distribution and dispersal, not limited to fluorochemical industrial areas. In particular, water sampling data allowed to estimate a 2.4 tons of GenX are discharged in the ocean every year from Chinese rivers (Pan et al., 2018).

Moreover, data from blood monitoring studies showed that high levels of exposure to GenX can be detected in both the exposed workers and the local population near fluoro-chemical plants (Gebbink and van Leeuwen, 2020). The increasing use of GenX for the manufacture of numerous tools to which people are exposed in ordinary life (Teflon pans and cookware, fire foams, paints and varnishes, detergents, and substances used in the photographic industry) implies that also the general population could be exposed to significant amounts of this compound (Coperchini et al., 2017).

Although GenX was introduced as a PFOA substitute, it was evidenced a greater toxicity of GenX as compared with PFOA and PFOS by recent studies (Gebbink et al., 2017; Gomis et al., 2018). Indeed, in vitro data showed that GenX activated the Peroxisome-proliferator-activated receptor alpha (PPARα) pathway and induced cell injury in spheroids of mouse liver cell lines (Sun et al., 2019). Furthermore, GenX was also shown to be hepatotoxic in animal models (Sheng et al., 2018) (Blake et al., 2020) and also could induce neoplastic transformation in the liver of female rats (Caverly Rae et al., 2015). A study comparing PFAS toxicities in a mouse model of hepatic damage showed that GenX was even more toxic than PFOA below the correction for differences in toxicity kinetics (Gomis et al., 2018). Moreover, similar adverse maternal, embryo and placental effects were observed in CD1 mice treated either with PFOA and GenX (Blake et al., 2020). Based on its environmental persistence and mobility, assessed by the fact that the compound was detected even faraway from its apparent source and also due to its proposed toxicity, in June 2019, the European Chemicals Agency (ECHA) identified GenX as a Substance of Very High Concern (SVHC) (Agency and documents, 2019).

In previous studies, we showed that PFOS and PFOA are detectable in specimens of thyroids derived from surgically operated. PFOS and PFOA are also able to enter thyroid cells after in vitro exposure on which also have a cytotoxic effect, even if at very high concentrations (Coperchini et al., 2015; Pirali et al., 2009). In a recent in vitro study, we found that four different short chain PFAS have no cytotoxic effects on thyroid cells and do not hamper with the control of thyroid function TSH-dependent (Croce et al., 2019). Up to now, no data regarding the potential toxicity of GenX on thyroid cells was reported in literature.

The aim of this study was to assess potential consequences of GenX exposure at different levels in thyroid cells. Based on the paucity of currently available in vitro data on GenX toxicity, we aimed at providing a wide spectrum of safety/toxicity data regarding the exposure to this novel compound by assessing cell viability and proliferation, changes in gene expression and DNA damaging effects which were all tested by treating rat thyroid cells with different concentrations of GenX.

2. Material and methods

2.1. Cultures of FRTL5 cells

FRTL-5 cells (ATCC CRL 8305, F1 subclone) were used as biological substrate for the experiments according to a previously established protocol (Thomasz et al., 2015).

2.2. Cell viability assay, WST-1 assay

FRTL-5 cells were seeded in 96-well flat-bottom plates supplemented with GenX at the following concentrations: 0.0001; 0.001; 0.01; 0.1; 1 μg/ml. These concentrations were chosen based on the plasma concentration of GenX found in the exposed workers, from 0.001 to 0.169 μg/ml (Conley et al., 2019; DuPont, 2017). Three incubation times were investigated: 24, 48 and 72 h. At the end of incubation with GenX, cell viability was detected by water-soluble tetrazolium salt (WST-1) (Coperchini et al., 2019).

2.3. Cell proliferation assay

To evaluate the ability of FRTL-5 cells surviving from GenX treatment to proliferate we incubated FRTL-5 cells with/out increasing concentrations of GenX (0.0001; 0.001; 0.01; 0.1; 1 μg/ml) for 24, 48 and 72 h. At the end of individual exposure time, cells were processed in according with previous established protocol (Crowley et al., 2016). More details in Supplementary Material and Methods.

2.4. Comet Assay

Genotoxicity was evaluated using an alkaline Comet Assay (Burlinson, 2012; Yang et al., 2019). Briefly cells were exposed to 0.0001 μg/ml and 0.001 μg/ml of GenX for three time points (24-48-72 h). Frosted microscope slides were dipped into hot 1% normal-melting-point agarose in PBS to the frosted area. Subsequently a cell suspension of 2×10^4 cells was mixed with 0.7% low-melting-point agarose and placed on the first agarose layer under a glass cover. After lysis in the appropriate lysis solution, slides were incubated in the appropriated denaturing buffer (More details in Supplementary Material and Methods). Next electrophoresis for slides was conducted for 15 min at 300 mA/25 V. Propidium Iodide

(PI) 20 μg/ml was added to slides after their immersion in the appropriate neutralization buffer (More details in Supplementary Material and Methods) and their draining. Images were obtained by using a fluorescence microscope as described in Supplementary Material and Methods.

2.5. Micronuclei assay

To confirm the genotoxicity of GenX, chromosome and chromatin damage after GenX exposure was assessed through micronucleus evaluation (Eke et al., 2017). After a 24-h incubation period with GenX (0.0001 μg/ml and 0.001 μg/ml), FRTL-5 cells processed and analysed in according with the established protocol (Eke et al., 2017). More details in Supplementary Material and Methods.

2.6. Real time PCR

FRTL-5 cells were treated with 0.0001 μg/ml of GenX for 24 h and then total RNA was processed and analysed in triplicate as previously described(Denegri et al., 2012). Pre-designed primers of TTF-1, PAX8, TG, TSHR, NIS and TPO were chosen based on the study by Lee et al. (2017).

2.7. Statistical analysis

Statistical analysis was performed by SPSS (SPSS, Inc., Evanston, IL). Mean values were compared by one-way ANOVA for normally distributed variables. *Post hoc* analysis was then performed by the Bonferroni's correction for multiple comparisons. Kruskall-Wallis test was used to compare variables with a non-parametric distribution as assessed through positivity to Levene test of equality of variances. *Post-hoc* analysis was then performed by the Dunn-Bonferroni's correction for multiple comparisons. Values are reported as mean ± SD unless otherwise noted. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of GenX on thyroid cell viability

To assess changes in FRTL-5 viability, a time-course incubation experiment was performed. Cells were exposed to GenX for an incubation time of 24–48 and 72 h. As shown in Fig. 1 (Panel A-B-C), exposure to GenX reduced FRTL-5 cells viability in a dose and time-dependent manner. Incubation with GenX reduced the viability of cells after 24 h (ANOVA $F = 22.286$; $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 μg/ml starting from GenX 0.001 μg/ml) (Fig. 1; Panel A) after 48 h (ANOVA $F = 43.253$, $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 μg/ml starting from GenX 0.001 μg/ml) (Fig. 1; Panel B) and after 72 h (ANOVA $F = 49.708$, $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 μg/ml starting from GenX 0.0001 μg/ml) (Fig. 1; Panel C).

3.2. Effect of GenX on cell proliferation of survived FRTL-5

To further investigate the cytotoxic effect of GenX we evaluated the proliferation ability of the cells that survived after GenX exposure by a cell proliferation assay. The results showed that survived FRTL-5 cells proliferation rate was reduced throughout the time course. In details: proliferation rate was reduced after 24 h (ANOVA $F = 11{,}941$; $p < 0{,}001$; Post-hoc vs untreated $p < 0.05$ for GenX 0.01 μg/ml and, 0.1 μg/ml and 1 μg/ml) (Fig. 5, Panel A); 48 h (ANOVA $F = 93.11$; $p < 0.001$; Post-hoc vs untreated $p < 0.05$ for GenX 0.0001 μg/ml, 0.001 μg/ml, 0.01 μg/ml and, 0.1 μg/ml and 1 μg/ml) (Fig. 5, Panel B) and 72 h (ANOVA $F = 21.65$; $p < 0.001$; Post-hoc vs untreated $p < 0.05$ for GenX 0.0001 μg/ml, 0.001 μg/ml, 0.01 μg/ml and, 0.1 μg/ml and 1 μg/ml) (Fig. 5, Panel C) of GenX exposure.

3.3. Genotoxic effect of GenX

To investigate the potential genotoxic effect of GenX exposure, the level of nuclear DNA integrity was assessed by comet assay under alkaline electrophoresis condition. The degree of the migration of DNA was increased in FRTL-5 cells after exposure to GenX (0.0001, 0.001 μg/ml) in a significant and time and dose-dependent manner. A representative image for each concentration of GenX exposure is shown in Fig. 2 (panels A-B-C). The comet assay data analysis was represented as tail length, DNA percentage and olive tail moment (all parameters expressed as logarithm) in the corresponding graphs (Fig. 2, panels D-E-F). GenX exposure increased all the tested parameters at all time points as shown in Fig. 2 in which the statistical significance is also shown. An increase of tail length, DNA percentage and olive tail moment was observed during the time course. An increase which was statistically significant in the levels of the three variables for each GenX concentration was observed at 48 and 72 when compared to 24 h.

To confirm data obtained with comet assay, we further investigated the genotoxic damage caused by GenX exposure by a micronucleus assay. In this assay, the percentage of micronuclei significantly increased after incubation with GenX (0.0001, 0.001 μg/ml) (Kruskall-Wallis $\chi2 = 23.962$, $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml) confirming the potential genotoxic effect of the compound (Fig. 3).

Effect of GenX on the expression of thyroid transcription factor genes and in genes involved in thyroid hormone synthesis.

The expression levels of two critical thyroid transcription factor genes (TTF-1 and Pax8) were evaluated. GenX, at non-cytotoxic concentrations, significantly reduced the expression of TTF-1 ($p < 0.05$) and increased the expression of Pax 8 ($p < 0.05$) (Fig. 4). We then evaluated a panel of genes involved in thyroid hormone synthesis. Only the expression of the Sodium-Iodide Symporter (NIS) gene was significantly inhibited after GenX exposure ($p < 0.05$). On the other hand, GenX did not affect the transcriptional expression levels of Thyroglobulin (Tg) ($p = 0.21$), TSH-receptor (TSH-R) ($p = 0.94$), and thyreoperoxidase (TPO) ($p = 0.604$) genes (Fig. 4).

4. Discussion

The results of this in vitro study represent the first demonstration that the exposure of differentiated rat thyroid cells (FRTL-5) to GenX produces acute cyto-toxic and geno-toxic effects. Indeed, GenX caused a time-and dose-dependent reduction in FRTL-5 cells viability. When the fate of the FRTL-5 cells surviving to GenX exposure was investigated, a significantly lower proliferation rate, as compared to never exposed cells, was found. These findings support the concept that the detrimental effect of GenX on FRTL-5 cell viability is not limited to acute toxicity, but produces a long-lasting damage, which persists even after the exposure to this compound has been relieved. Further to these cytotoxic effects, GenX also jeopardized the DNA integrity of FRTL-5 cells, both at cytotoxic and non-cytotoxic concentrations. Indeed, in the comet assay, GenX increased in a dose and time-dependent manner the formation of comets. In accordance with these findings, exposure to GenX resulted in the appearance of micronuclei in FRTL-5 cells.

GenX also modulated the expression of two pivotal genes implicated in thyroid organogenesis and of the Sodium-Iodide symporter (NIS). GenX reduced the TTF-1 expression, a gene involved in the embryologic formation of the thyroid (De Felice and Di Lauro, 2004) and, when expressed at low level, in the promotion

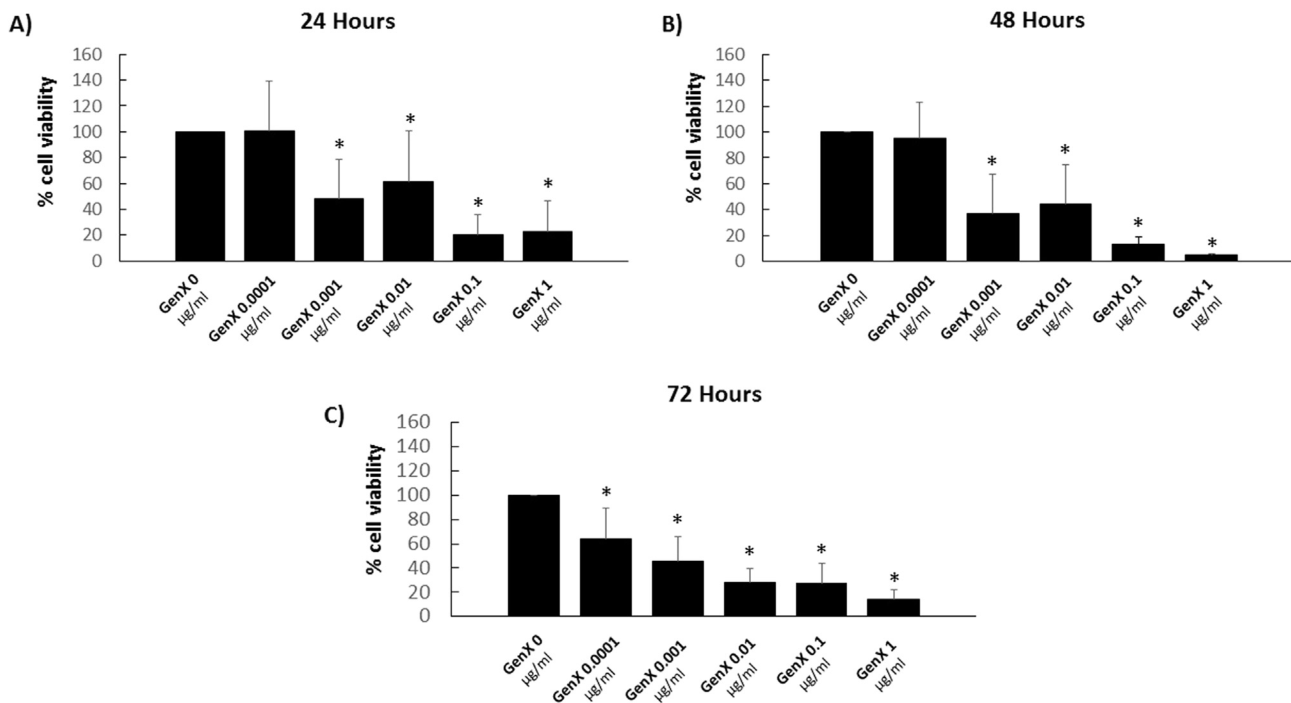


Fig. 1. Effect of GenX on FRTL-5 viability. *Panel A:* Incubation with GenX reduced cell viability following 24 h (ANOVA F = 22.286; $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 µg/ml starting from GenX 0.001 µg/ml). *Panel B:* incubation with GenX reduced cell viability at 48 h (ANOVA F = 43.253, $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 µg/ml starting from GenX 0.001 µg/ml) *Panel C:* Incubation with GenX reduced cell viability at 72 h (ANOVA F = 49.708, $p < 0.001$; Post Hoc $p < 0.05$ vs. GenX 0 µg/ml starting from GenX 0.0001 µg/ml). Results expression as % of viable cells calculated on the OD of untreated samples estimated as 100%.

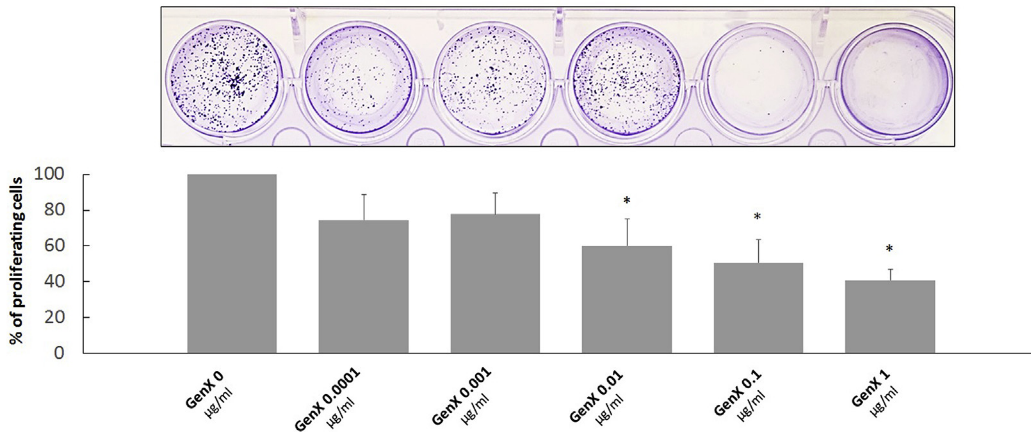
of apoptosis. On the other hand, GenX up-regulated the expression of Pax8, a master gene for the regulation of thyroid differentiation (De Felice and Di Lauro, 2004). Pax8 expression is needed for thyroid precursor cells survival, but it also checks the development of thyroid gland and its functional differentiation (De Felice and Di Lauro, 2004). Although the combined effect of Genx on thyroid transcription factors genes remains to be investigated in *in vivo* models, our findings strongly agree with the hypothesis that in utero exposure to this compound might disrupt thyroid organogenesis. The Pax8 gene is also overexpressed in thyroid cancer, and it is thus suspected to play a role in the early events of thyroid carcinogenesis (Dupain et al., 2016). The fact that GenX up-regulates the Pax8 gene expression suggests a pro-tumorigenic effect of this compound in adult life.

Lastly, the down-regulation of the NIS gene, responsible for the uptake of iodide in thyroid gland (Yu et al., 2009; Zoeller, 2007),is in line with the endocrine-disrupting effects observed after exposure of FRTL-5 to legacy PFAS like PFHxS and PFOS (Buckalew et al., 2020) and PFDoA (Zhang et al., 2018) and to several other compounds, like bisphenols (Wu et al., 2016). A schematic diagram (Fig. 6) summarizes the end-points and results of the present study.

Our in vitro findings raise further concerns on the possible detrimental effects of GenX on animal and human health. GenX is a widespread pollutant, reaching very high concentrations not only in environmental samples, but also in the plasma of exposed workers (Conley et al., 2019; DuPont, 2017; Gomis et al., 2018). In view of its persistency and mobility in the aquatic environment and its continuous emission, recently the European Chemicals Agency (ECHA) identified GenX as a Substance of Very High Concern (SVHC) (Agency and documents, 2019). With specific regards to this point, it should be highlighted that our results were obtained using Genx concentrations, which are encompassed in the range of those found in exposed workers sera (DuPont, 2017). GenX was engineered to substitute long-chain PFAs (such as PFOA and PFOS) with the aim to reduce bioaccumulation and risks for human health (Buck et al., 2011; Gomis et al., 2018; Heydebreck et al., 2015a; Wang et al., 2019), but the results of the present study would indicate that, at least on rat thyroid cells, GenX could exert adverse effects.

The present study was not aimed at comparing the toxicity profile between GenX and either legacy long-chain PFAs (such as PFOA and PFOS) nor the other numerous short-chain PFAs currently employed. However, based on the available data, some considerations could be drawn. First of all, GenX has a chemical structure which in some ways can be assimilated to the family of PFAs conferring it the peculiar features of these compounds (DuPont, 2017; Pica et al., 2019). Nevertheless, the new congeners of PFAS (such as GenX) developed in recent years have slightly different structural characteristics when compared with historical PFAS such as PFOA and PFOS, and thus their effects could be different.

On the other hand, several toxic effects we observed are not typical of other PFAs. Previous studies failed to show any genotoxic effect of PFOA and PFOS (Temkin et al., 2020), while GenX showed significant genotoxicity in our study, as assessed by both comet assay and micronucleus assay. Indeed a 2010 study by Eriksen et al. specifically aimed at studying the potential genotoxicity of different PFAS, failed to show any DNA damage through comet assay in HepG2 cells treated with PFOA, PFOS, PFBS and PFHxA, with only a limited effect of PFNA (Eriksen et al., 2010). Also when the endocrine-disruptor activity of PFAs is considered, GenX appears to have a peculiar toxicity profile. For example, a very recent study addressing the effects of gestational exposure to GenX and PFOA in CD-1 mice showed that GenX recapitulated many documented effects of PFOA, regardless of its much shorter reported half-life, but some of the placental adverse effects seemed to differ between the two PFAS (for example, PFOA exposure caused labyrinth congestion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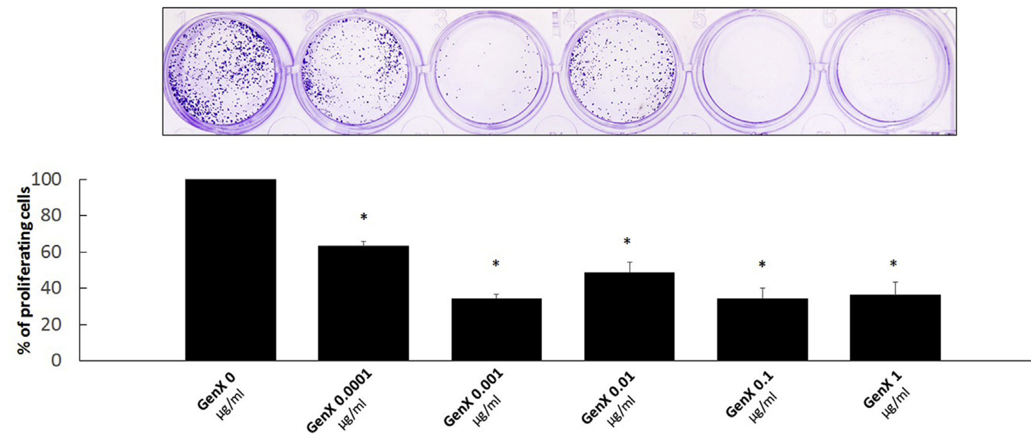
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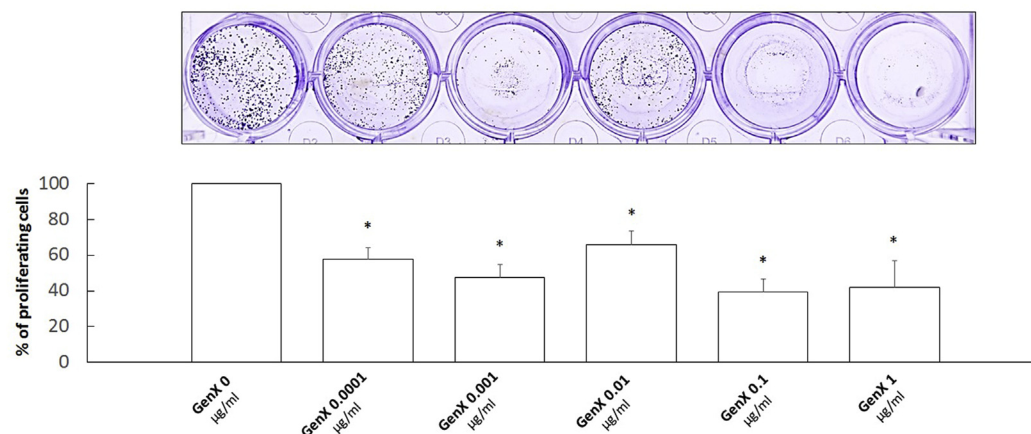
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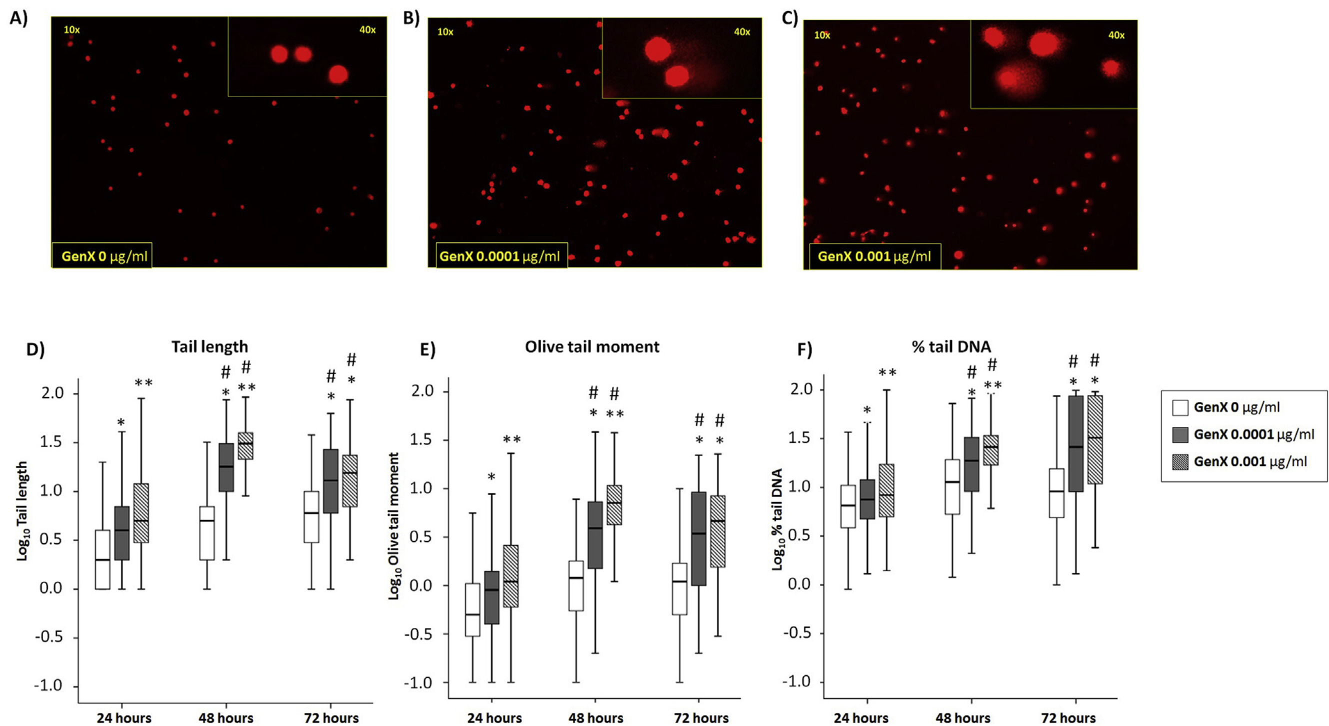


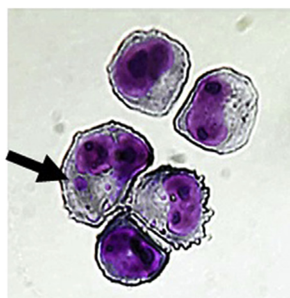
Fig. 3. Results of Comet Assay. *Panel A-B-C*: representative images of comet assays performed on FRTL-5 incubated with to GenX 0 μg/ml, GenX 0.0001 μg/ml and GenX 0.001 μg/ml, evidencing DNA comet formation in cells exposed to GenX. *Panel D:* Tail length values in cells exposed to GenX 0 μg/ml, GenX 0.0001 μg/ml and GenX 0.001 μg/ml after 24 h (Kruskall-Wallis $\chi2 = 133.8$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml; ** $p < 0.05$ for GenX 0.001 μg/ml vs 0.0001 μg/ml), 48 h (Kruskall-Wallis $\chi2 = 86.2$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml; ** $p < 0.05$ for GenX 0.001 μg/ml vs 0.0001 μg/ml) and 72 h (Kruskall-Wallis $\chi2 = 28.9$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml). In addition, tail length increased also over time for both GenX 0.0001 μg/ml (Kruskall-Wallis $\chi2 = 214.4$, $p < 0.001$; Post Hoc #$p < 0.05$ vs. 24 h for 48 h and 72 h treatment) and GenX 0.001 μg/ml (Kruskall-Wallis $\chi2 = 199.5$, $p < 0.001$; Post Hoc #$p < 0.05$ vs. 24 h, 48 h and 72 h treatment). *Panel E:* Olive tail moment values in cells exposed to GenX 0 μg/ml, GenX 0.0001 μg/ml and GenX 0.001 μg/ml at 24 h (Kruskall-Wallis $\chi2 = 74.1$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml; ** $p < 0.05$ for GenX 0.001 μg/ml vs 0.0001 μg/ml), 48 h (Kruskall-Wallis $\chi2 = 63.7$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml; ** $p < 0.05$ for GenX 0.001 μg/ml vs 0.0001 μg/ml) and 72 h (Kruskall-Wallis $\chi2 = 38.7$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml). In addition, olive-tail-moment increased also over time for both GenX 0.0001 μg/ml (Kruskall-Wallis $\chi2 = 151.3$, $p < 0.001$; Post Hoc #$p < 0.05$ vs. 24 h, 48 h and 72 h treatment) and GenX 0.001 μg/ml (Kruskall-Wallis $\chi2 = 146.1$, $p < 0.001$; Post Hoc #$p < 0.05$ vs. 24 h for 48 h and 72 h treatment). *Panel F:* Percentage of tail DNA values in cells exposed to GenX 0 μg/ml, GenX 0.0001 μg/ml and GenX 0.001 μg/ml after 24 h (Kruskall-Wallis $\chi2 = 27.7$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml; ** $p < 0.05$ for GenX 0.001 μg/ml vs 0.0001 μg/ml), 48 h (Kruskall-Wallis $\chi2 = 26.1$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml; ** $p < 0.05$ for GenX 0.001 μg/ml vs 0.0001 μg/ml) and 72 h (Kruskall-Wallis $\chi2 = 41.4$, $p < 0.001$; Post Hoc *$p < 0.05$ vs. GenX 0 μg/ml for GenX 0.0001 and 0.001 μg/ml). In addition, percentage of tail DNA olive-tail-moment increased also over time for both GenX 0.0001 μg/ml (Kruskall-Wallis $\chi2 = 102.2$, $p < 0.001$; Post Hoc #$p < 0.05$ vs. 24 h for 48 h and 72 h treatment) and GenX 0.001 μg/ml (Kruskall-Wallis $\chi2 = 94.6$, $p < 0.001$; Post Hoc #$p < 0.05$ vs. 24 h for 48 h and 72 h treatment). Values are expressed as Log_{10} of raw data. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers.

while GenX exposure more often caused labyrinth atrophy) (Blake et al., 2020). Moreover, a recent study addressing the impact of PFOA and GenX on gene expression and epigenetic modifications on hepatocellular carcinoma cells showed that the two PFAs exerted markedly different actions on several lipid metabolism related genes (Wen et al., 2020). These data, although still limited and obtained in different experimental settings, would support the concept that a significant difference in the endocrine disruptive potential of GenX exists when compared to other PFAs.

The design of the present study does not allow drawing conclusions on the biochemical mechanisms by which GenX induces the here described effects. However, previous studies indicated that the toxicity profile of GenX would suggest a similar effect of some PPARα activators (Gordon, 2011; Thompson et al., 2019; Wang et al., 2017). PPARα is a nuclear receptor that binds with peroxisome-proliferator-response-elements in the promoter region of target genes, influencing lipid catabolism, fatty acid uptake and lipoprotein transport and assembly (Pyper et al., 2010). A recent study by Chappell et al. reported that liver changes observed in GenX-treated mice (hypertrophy and apoptosis) occur via a mode of action involving PPARα (Chappell et al., 2020). However the PPAR pathway is not probably the only mechanism affected by GenX exposure. Indeed in the recent study by Wen et al. (2020) it was found that GenX reduced HepG2 cells viability through a complex mechanism involving epigenetic alterations in gene methylation of some essential lipid metabolism genes and disruption of the physiological cell cycle. These preliminary information, highlight the need for future studies specifically designed to characterize the potential mechanisms involved in GenX toxicity.

Although data regarding the direct cytotoxic effect of GenX are worrisome, because they suggest a possible involvement of GenX exposure in pathogenesis of hypothyroidism, an even greater

Fig. 2. Assay of cell proliferation on survived cells following exposure to GenX. Panel A-B-C show histograms and representative images of the cell proliferation assay. *Panel A:* the ability of survived FRTL-5 cells to proliferate was reduced after 24 h of GenX exposure at increasing concentrations (ANOVA $F = 11{,}941$; $p < 0{,}001$; *Post-hoc vs GenX 0 μg/ml $p < 0.05$ starting from GenX 0.01 μg/ml). *Panel B:* the ability of survived FRTL-5 cells to proliferate was reduced after 48 h of GenX exposure at increasing concentrations (ANOVA $F = 93.11$; $p < 0.001$; *Post-hoc vs GenX 0 μg/ml $p < 0.05$ starting from 0.0001 μg/ml). *Panel C:* the ability of survived FRTL-5 cells to proliferate was reduced at 72 h of GenX exposure at increasing concentrations (ANOVA $F = 21.65$; $p < 0.001$; *Post-hoc vs GenX 0 μg/ml $p < 0.05$ starting from 0.0001 μg/ml).



	% MICRONUCLEI PER FIELD	N. MICRONUCLEI/1000 CELLS	p Value
GenX 0 µg/ml	0.0629±0.35	0.54	
GenX 0.0001 µg/ml	0.7086±1.27	8.34	p<0.005
GenX 0.001 µg/ml	1.1126±1.81	18.10	p<0.0001

Fig. 4. Representative image of micronucleus (FRTL-5 cells treated for 24 h with GenX 0.001 µg/ml). Micronucleus assay: FRTL-5 cells were exposed for 24 h to GenX 0.0001 µg/ml and 0.001 µg/ml. Kruskall-Wallis test $\chi2 = 23.962$, $p < 0.001$. *post-hoc analysis vs GenX 0 µg/ml.

preoccupation is related to the possible disrupting effect on in utero thyroid organogenesis and to its genotoxicity. Indeed, it might be hypothesized that cells surviving to GenX exposure suffer a DNA damage that in the long run could favour the development of thyroid cancer. This finding would be in line with early reports regarding the induction of tumorigenic pathways in mice exposed to GenX (Conley et al., 2019; Sheng et al., 2018).

These data, although preliminary, should raise concern regarding the potential toxicity of a compound increasingly released in the environment. Future studies using other cell types and/or animal models will be required to confirm these findings and to shed some light on the physiopathological mechanisms underlying GenX toxicity. Furthermore, we recommend that a close monitoring of the potential endocrine-disrupting effects of GenX should be performed on exposed population (including exposed workers and people living near fluorochemical plants). Indeed, the European Chemical Agency (ECHA) recently recommended further studies, including a biomonitoring study of volunteer workers at GenX-producing plants, and a carcinogenicity study in mice.

In conclusion, the present study demonstrated the potential thyroid toxicity of GenX, which appears to be far more relevant than the one of any other PFAS (both short and long-chain) investigated so far in thyroid cells.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have

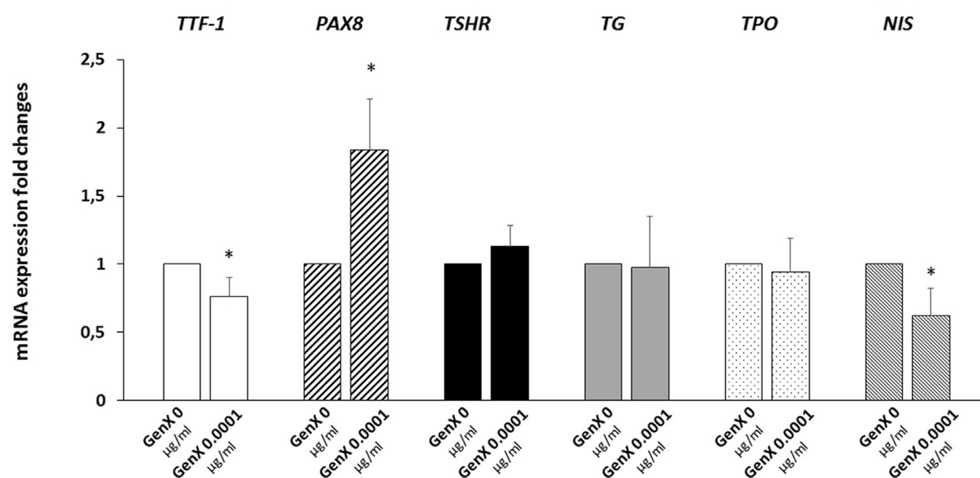


Fig. 5. Relative mRNA expression of a selected panel of genes playing a role in thyroid hormone synthesis and transcription factor genes during GenX exposure. FRTL-5 cells were incubated with GenX 0.0001 µg/ml for 24 h. Quantitative data were normalized to expression levels of untreated cells and related to expression in GenX 0.0001 µg/ml exposed cells. No changes significant in the expression of Tg (Student's T test $F = 15.01$; $p = 0.21$), TSH-R genes (Student's T test $F = 7.4$; $p = 0.94$) and TPO genes (Student's T test $F = 0.55$; $p = 0.60$) were detected in exposed cells. GenX reduced significantly TTF-1 expression (Student's T test $F = 13.7$; $p < 0.05$) and NIS expression (Student's T test $F = 4.17$; $p < 0.05$), while it increased Pax8 expression (Student's T test $F = 15.15$; $p < 0.05$).

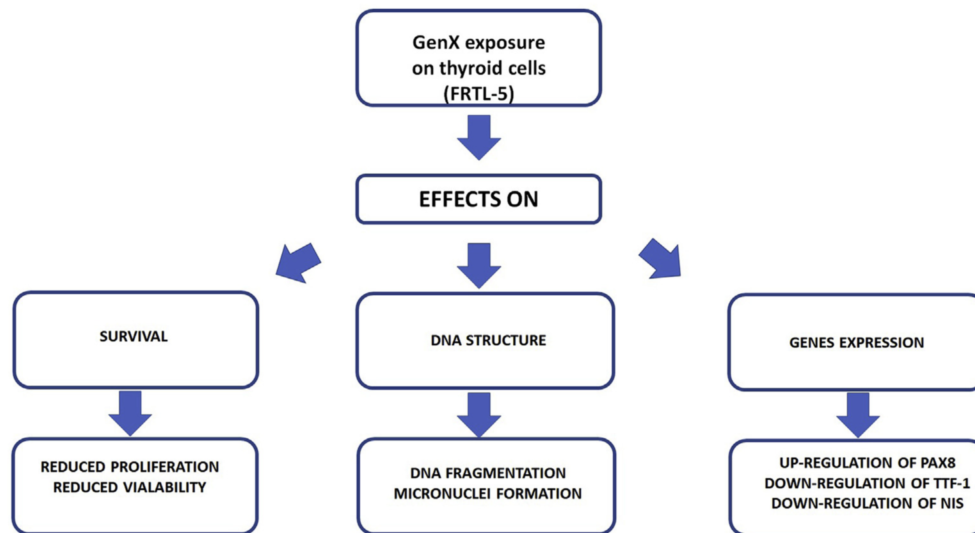


Fig. 6. Schematic diagram summarizing the end-points and results of the present study. GenX exerted adverse effects on FRTL-5 thyroid cells at different levels: 1) reduction of cell proliferation and viability; 2) alteration of DNA structure by inducing DNA fragmentation (as showed by comet assay) and formation of micronuclei; 3) Modulation of the expression of some thyroid-related genes (up-regulation of PAX8, down-regulation of NIS and TPO expression).

appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Francesca Coperchini: Conceptualization, Methodology. **Laura Croce:** Writing - original draft, Software. **Marco Denegri:** Methodology, Writing - original draft. **Patrizia Pignatti:** Formal analysis, Writing - review & editing, Supervision. **Manuela Agozzino:** Supervision. **Giuseppe Stefano Netti:** Software, Formal analysis. **Marcello Imbriani:** Writing - review & editing. **Mario Rotondi:** Writing - review & editing. **Luca Chiovato:** Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114778.

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